61 can be found in Claims 1-21, as originally filed, and on page 26, line 16, to page 27, line 10.

No new matter has been added. Claims 23-61 are active in this application.

REMARKS

At the outset, Applicants' representative wishes to thank Examiners Nyugen and Guzo for the helpful and courteous discussion held on June 25, 2001, during which the prosecution of the above-identified application was materially advanced, and it was agreed that the requirement for a substitute specification set out the Official Action dated March 15, 2001, was withdrawn. The following remarks will expand and summarize the issues discussed.

The rejection of Claims 1-4 and 17 under 35 U.S.C. § 102(b) in view of Friedman et al and Nakamura et al and the rejection of Claims 1-4 and 17 under 35 U.S.C. § 102(e) in view of published PCT application WO 95-19949 (Friedman et al) or U.S. Patent No. 6,162926 (Murphy et al) have been obviated by appropriate amendment. As the Examiner will note Claim 17 has been canceled and Claims 1-4 have been rewritten as Claims 23-33, which are directed toward methods for compacting DNA. None of the cited references are at all concerned with compacting DNA and are thus not relevant to new Claims 23-33.

Accordingly, these rejections are no longer tenable and should be withdrawn.

The rejection of Claims 1-16 and 18-21 under 35 U.S.C. § 112, second paragraph, and the rejection of Claims 1-10 and 21 under 35 U.S.C. § 101 have been obviated by appropriate amendment. As the Examiner will note, Applicants have rewritten the claims such that they are free of the criticisms outlined on pages 4-6 of the Official Action.

Once again, the rejections are no longer proper and should be withdrawn.

The objection to Claims 2, 4, 6, 8, 18, and 20 as containing parentheses has been obviated by rewriting the claims to delete all parentheses except those require by the rules of chemical nomenclature. Similarly, the objection to Claims 11 and 14 as containing brackets has been obviated by rewriting the claims to delete all unnecessary brackets.

Thus, these objections should be withdrawn.

Applicants wish to thank Examiner Nyugen for bringing to their attention the existence of A. M. Cassell et al., Angew. Chem. Int. Ed., vol. 37, pp. 1528-1531 (1998) (Cassell et al.) during the above-noted interview. Although the PTO has not issued any rejection of any claims over Cassell et al., Applicants submit that this reference cannot affect the patentability of the present claims for the following reasons. In particular, the Examiner's attention is directed toward H. Isobe et al., Chemistry Letters, 2001, pp. 1214-1215 (Isobe et al.) a copy of which is being filed herewith, as Exhibit A. In Isobe et al., a direct comparison of the compound of Cassell et al. (compound 3 in Isobe et al.) with a compound according to the present claims (*e.g.*, compound 1 in Isobe et al.) is presented. As reported in the paragraph bridging the cols. on page 1215 of Isobe et al., the compound according to the present claims exhibited superior transfection properties as compared to the compound of Cassell et al. Applicants respectfully request that the Examiner consider the results presented in Isobe et al., before deciding to issue any rejection based on Cassell et al.

Applicants submit that the application is now in condition for allowance, and early notification of such action is earnestly solicited.

Respectfully submitted,

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Amendment Filed on:
December 17, 2001

IN THE CLAIMS

Please cancel Claims 1-22, without prejudice toward the further prosecution of these claims in a continuation and/or divisional application.

Please add the following new claims:

--23. (New) to 61. (New)--

Synthesis and Transf ction Capability f Multi-Functionalized Fullerene P lyamine

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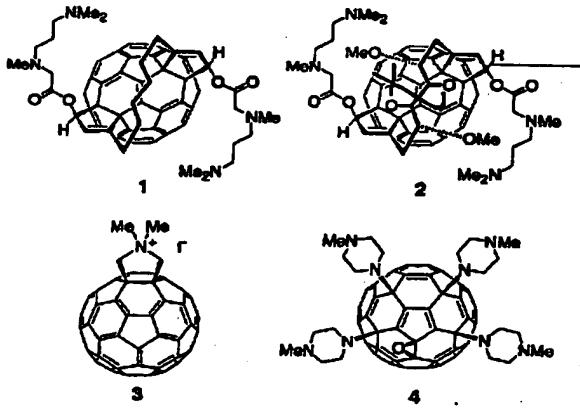
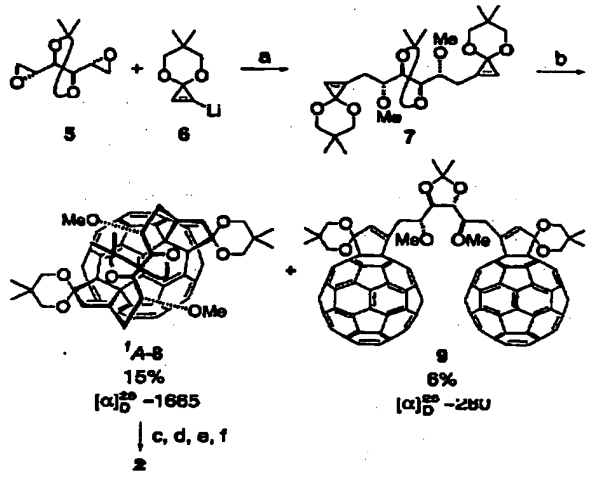
A new fullerene transfection reagent bearing multiple-functional groups has been synthesized by diastereoselective double cycloaddition reaction. The highly oxygenated reagent transfers extra-cellular DNA into mammalian cells with the efficiency comparable to that of a nor-analogue.

Interaction of organofullerenes and DNA has attracted considerable interest of chemists since the report that a water-soluble fullerene cleaves DNA under irradiation with visible light.[1,2] Recently an entirely new possibility of fullerene biology was pointed out—transfection of mammalian cells.[3] Thus, the fullerene tetramine **1** was found to be able to bind to DNA and to deliver the bound DNA into mammalian cells with efficiency comparable to conventional lipid-based transfection agent. This prototype reagent **1** embodies a very simple design principle possessing two sets of trimethylene diamine side chains, which are separated by 1.2 nm from each other so that they may have maximum electrostatic interaction with the two phosphate side chains f double strand DNA. To further develop the fullerene-mediated transfection technology, we felt it necessary to study the installation of functional sites, on which additional functional groups such as base-recognition groups may be attached. To this end, we investigated the synthesis of **2**, which is a highly oxygenated analogue of **1**, and its transfection ability to examine whether or not such heavy substitution on the hexamethylene moiety of **1** affects the biological activity. The transfection capability of **2** was found to be of the same order as **1**, suggesting that installation of elaborate functionality on the hexamethylene tether is tolerable. Two other DNA-binding fullerenes **3** and **4** did not show any transfection ability, which demonstrates that the electrostatic interaction with DNA is not the only structural requirement for the fullerene-transfection reagents, but suitable spatial arrangement of the functional groups is crucial.

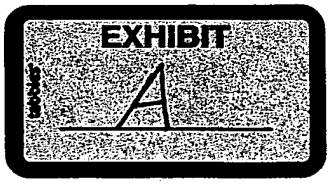


We started the synthesis of **2** with the known diepoxide **5**[4] (prepared from D-mannitol), which was allowed to react with the lithiated cyclopropenone acetal **6**[5] to obtain the bis-cyclopropenone acetal **7**. Diepoxide **5** was added to a THF solution f **6** at –40 °C and the mixture was stirred for 20 h. To the reaction mixture was added 10 equiv of MeI and the reaction mixture was warmed to room temperature. Double addition of **6** and double methylation of the resulting alkoxide took place in one pot to give the desired biscyclopropenone acetal **7** in 45% yield after silica gel column chromatography.



Scheme 1. (a) HMPA, THF, –40 °C, 20 h, then MeI, rt, 2 h, 46%; (b) C_{60}, degassed 1,2-$Cl_2C_6H_4$, 150 °C, 7 d; (c) H_2SO_4, H_2O, THF, PhCl, 50 °C, 21 h, 64%; (d) DIBAL-H, PhCl, –25 °C, 14 min, 82%; (e) $BrCH_2COBr$, pyridine, DMAP, PhCl, rt, 6 h, 73%; (f) $HNMe(CH_2)_2NMe_2$, degassed PhCl, dark, rt, 5 h, 23%.

In the next stage, **7** was heated with [60]fullerene (C_{60}) to obtain regioselectively the double cycloaddition product **8**. In the previous report, we used a nor-analogue of **7** that lacks the oxygen substituents, and carried out the reaction at a relatively low concentration (1.5 mmol·L^{-1}) to obtain a racemic single regioisomer in 41% isolated yield.[6] Under the same reaction conditions, the desired double cycloadduct **8** did not form at all, and 85% of unreacted C_{60} was recovered even after 11 d. Such low reactivity of **7** is likely due to both steric and electronic effects of the substituents in **7**. The reaction was therefore carried out for 7 d at a higher concentration (14 mmol·L^{-1} in C_{60}) in a sealed glass tube which was previously treated with *N,O*-bis(trimethylsilyl)acetamide (Scheme 1). Purification with silica gel column chromatography afforded the desired double cycloadduct *f*-**8**[7] in 15% yield. No other diastereomeric double adduct was detected in th product



mixture. FABMS spectrum was consistent with the 1:1 adduct. Unreacted C_{60} was recovered in 42% yield and 1:2 double cycl adduct 9 was also obtained in 6% yield. ^{f}A-8 was subsequently converted to tetramine 2 in 4 steps (Scheme 1) by the synthetic route reported for tetramine 1.[3a]

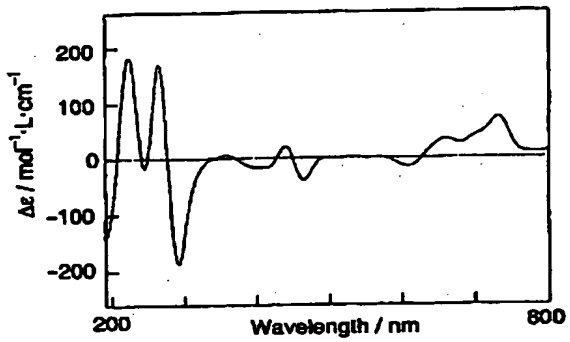


Figure 1. CD spectrum of ^{f}A-6 (7.0 μmol·L^{-1} in cyclohexane).

Stereochemical assignment of ^{f}A-8 needed considerable care. ^{1}H NMR and ^{13}C NMR indicated that the double cycloadduct is a single isomer of C_2-symmetry. Very large optical rotation of the product ($[\alpha]_D$ −1665°, c = 0.01, $CHCl_3$, 28 °C) is characteristic to non-racemic fullerenes produced by chiral double cycloaddition.[6a] The optical rotation of 1:2 adduct 9 was much smaller ($[\alpha]_D$ −280°, c = 0.01, $CHCl_3$, 28 °C). In spite of previous efforts[8] to determine the absolute configuration of such double cycloaddition products, no conclusive method of structural assignment has been obtained. In an attempt to assign the stereochemistry of our double cycloadduct 8, we performed molecular mechanics analysis[6b,9] to find that ^{f}A diastereomer is slightly more stable than the ^{f}C diastereomer (by 0.41 kcal/mol). The CD spectrum of 8 showed strong positive Cotton effect in the region of 210–230 nm (Figure 1). Such positive Cotton effect was also observed for a simpler analogue of 8[6] as well as for a fullerene bismalonate, which was proposed to have ^{f}A configuration deduced from theoretical analysis of CD spectra.[8]

DNA-binding ability of tetramine 2 was then examined with competitive binding assay using ethidium bromide.[10] In order to investigate the structure–activity relationship in the transfection ability, two other fullerenes 3[11] and 4[12] were examined as reference. The compound 3 showed moderate binding ability (C_{50} = 2.8 μmol·L^{-1}), and 2 (C_{50} = 1.6 μmol·L^{-1}) and 4 (C_{50} = 1.2 μmol·L^{-1}) showed comparable binding ability as our previous transfection reagent 1 (1.9 μmol·L^{-1}).[3] All compounds (1–4) strongly bind to DNA. In addition, the side chain substitution in two-handed fullerene did not interfere the binding of the molecule with DNA.

With this knowledge in hand, we carried out transfection experiments with the DNA binding fullerene under the optimized conditions (reagent/base pair = 13).[3] Each DNA binding fullerene (1 and 2) was mixed with plasmid DNA (pGreen LANTERN-1) that contains a reporter gene of green fluorescent protein (GFP). After 30 min, the resulting fullerene-DNA complex was added to cultured NIH 3T3 cells. After 2-day incubation, GFP expression in the transfected cells was observed with fluorescence microscope to find transfection efficiency of 2 to be 3.8×10^{-4}, which is comparable to the value obtained for 1 under the same conditions (2.1×10^{-4}). On the other hand, experiments with the other reference compounds 3 and 4 did not show any transfection capability.

In summary, we reported the second example of gene delivery with functionalized fullerene, and found that installati n of polar substituents does not hinder the binding of the fullerene 2 to DNA. This observation combined with the propensity of fullerene to form stable aggregates in water[13] suggest that, in the fullerene–DNA condensate obtained from 1 or 2, the molecule is located in such a way that the hydrophilic oxygenated moiety points toward the interior of the groove, while the unmodified fullerene core points outward so that it can maintain hydrophobic interaction with other fullerene molecule. With such a working model, one may expect that installation of a base-selective functionality on the oxygenated side chain will create a base-selective DNA binding agent. This intriguing possibility will be the subject of further studies.

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Dedicated to Prof. Hideki Sakurai on the occasion of his 70th birthday.

References and Notes

1 a) H. Tokuyama, S. Yamago, and E. Nakamura, *J. Am. Chem. Soc.*, 115, 7918 (1993). b) A. S. Boutorine, H. Tokuyama, M. Takasugi, H. Isobe, E. Nakamura, and C. Hélène, *Angew. Chem., Int. Ed. Engl.*, 33, 2462 (1994). c) E. Nakamura, H. Tokuyama, S. Yamago, T. Shiraki, and Y. Sugiura, *Bull. Chem. Soc. Jpn.*, 69, 2143 (1996). d) K. Irie, Y. Nakamura, H. Ohigashi, H. Tokuyama, S. Yamago, and E. Nakamura, *Biosci. Biotechnol. Biochem.*, 60, 1359 (1996). e) S. Yamago, H. Tokuyama, E. Nakamura, K. Kikuchi, S. Kananishi, K. Sueki, H. Nakahara, S. Enomoto, and F. Ambe, *Chem. Biol.*, 2, 385 (1995).

2 a) Y. -Z. An, C. -H. B. Chen, J. L. Anderson, D. S. Sigman, C. S. Foote, and Y. Rubin, *Tetrahedron*, 69, 2143 (1996). b) Y. Tabata, Y. Murakami, and Y. Ikada, *Jpn. Cancer Res.*, 88, 1108 (1997). c) Y. Yamakoshi, S. Sueyoshi, K. Fukuhara, and N. Miyata, *J. Am. Chem. Soc.*, 120, 12363 (1998). d) R. Bernstein, F. Prato, and C. S. Foote, *J. Am. Chem. Soc.*, 121, 464 (1999).

3 a) E. Nakamura, H. Isobe, N. Tomita, M. Sawamura, S. Jinno, and H. Okayama, *Angew. Chem. Int. Ed.*, 39, 4254 (2000). b) H. Isobe, S. Sugiyama, K. -i. Fukui, Y. Iwasawa, and E. Nakamura, *Angew. Chem. Int. Ed.*, 40, 3364 (2001).

4 Y. L. Merrer, A. Duréault, C. Greck, D. Micas-Languin, C. Gravier, and J. -C. Depezay, *Heterocycles*, 25, 541 (1987).

5 M. Isaka, S. Ejiri, and E. Nakamura, *Tetrahedron*, 48, 2045 (1992).

6 a) E. Nakamura, H. Isobe, H. Tokuyama, and M. Sawamura, *Chem. Commun.*, 1996, 1747. b) H. Isobe, H. Tokuyama, M. Sawamura, and E. Nakamura, *J. Org. Chem.*, 62, 5034 (1997). c) H. Isobe, M. Sawamura, and E. Nakamura, *Fullerene Sci. Technol.*, 7, 519 (1999).

7 See for nomenclature of chiral fullerenes: C. Thilgen, A. Herrman, and F. Diederich, *Helv. Chim. Acta*, 80, 183 (1997).

8 a) H. Goto, N. Harada, J. Crassous, and F. Diederich, *J. Chem. Soc., Perkin Trans. 2*, 1998, 1719. b) R. Kessinger, C. Thilgen, T. Mordasini, and F. Diederich, *Helv. Chim. Acta*, 83, 3069 (2000).

9 Molehar mechanics calculations with MM2* force field were performed with MacroModel ver 4.5. The total steric energies of the global minima were obtained by Monte Carlo conformation search (ref 6b).

10 B. F. Cain, B. C. Baguley, and W. A. Denny, *J. Med. Chem.*, 21, 658 (1978).

11 The reference compound 3 has been reported to bind DNA under electron microscopy conditions: A. M. Cassell, W. A. Scrivens, and J. M. Tour, *Angew. Chem. Int. Ed.*, 37, 1528 (1998).

12 H. Isobe, N. Tomita, and E. Nakamura, *Org. Lett.*, 2, 3663 (2000).

13 a) M. Sawamura, N. Nagahama, M. Toganoh, U. E. Hackler, H. Isobe, E. Nakamura, S. -Q. Zhou, and B. Chu, *Chem. Lett.*, 2000, 1098. b) S. Zhou, C. Burger, B. Chu, M. Sawamura, N. Nagahama, M. Toganoh, U. E. Hackler, H. Isobe, and E. Nakamura, *Science*, 291, 1944 (2001).